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Transposon Mutagenesis in *Actinobacillus pleuropneumoniae* with a Tn*10* Derivative

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A transposon mutagenesis procedure functional in the gram-negative swine pathogen *Actinobacillus pleuropneumoniae* was developed for the first time. The technique involved the use of a suicide conjugative plasmid, pLOF/Km, carrying a mini-Tn*10* with an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible transposase located outside the mobile element (M. Herrero, V. de Lorenzo, and K. N. Timmis, J. Bacteriol. 172:6557–6567, 1990). The plasmid was mobilized from *Escherichia coli* to *A. pleuropneumoniae* through the RP4-mediated broad-host-range conjugal transfer functions provided by the chromosome of the donor strain. When IPTG was present in the mating medium, *A. pleuropneumoniae* CM5 transposon mutants were obtained at a frequency of 10^{-5}, while no mutants were detected in the absence of IPTG. Since the frequency of conjugal transfer of the RP4 plasmid from *E. coli* to *A. pleuropneumoniae* CM5 was found to be as low as 10^{-4}, the above result indicated that the expression level of the transposase was a critical factor for obtaining a workable efficiency of transposon mutagenesis. The transposon insertions occurred at random, as determined by Southern blotting of chromosomal DNA of randomly selected mutants and by the ability to generate mutants defective for the selected phenotypes. Almost all the mutants analyzed resulted from a single insertion of the Tn*10* element. About 1.2% of the mutants resulted from the cointegration of pLOF/Km into the *A. pleuropneumoniae* chromosome. The applicability of this transposon mutagenesis system was verified on other *A. pleuropneumoniae* strains of different serotypes. The usefulness of this transposon mutagenesis system in genetic studies of *A. pleuropneumoniae* is discussed.

The gram-negative, facultatively anaerobic, encapsulated bacterium *Actinobacillus pleuropneumoniae* (formerly classified in the genus *Haemophilus*) is an important veterinary pathogen of the *Pasteurellaceae* family (23, 32). It is the etiologic agent of porcine pleuropneumonia, a highly contagious respiratory infection causing heavy economic losses to the swine industry (30, 38). Clinically, *A. pleuropneumoniae* infection occurs as acute outbreaks of high mortality in 24 to 48 h or as chronic persistent infections. The latter are of special concern because of their high prevalence and because they follow an insidious course, manifested by impairment of the growth rates of pigs in the fattening units (30, 38).

Currently available vaccines to *A. pleuropneumoniae* are empirical and are not satisfactory enough in controlling the disease in porcine populations. The major drawbacks of these vaccines include a lack of cross-protection between serotypes (12 have been described to date [20, 31]) and failure to prevent the spread of the chronic infection from carriers (18, 36). As a first step in the development of new efficient vaccines, considerable efforts and resources have been invested in research on the pathogenic and immunogenic mechanisms of *A. pleuropneumoniae*. Several putative virulence factors have been investigated (19), such as the serotype-specific capsular material (8, 19, 36), the lipopolysaccharide (2, 8), the surface proteins (33, 45), and the cytolytic protein toxins responsible for the hemolytic phenotype of *A. pleuropneumoniae* (9, 11, 12). The study of these putative virulence factors has been approached by means of purification and characterization techniques (2, 9, 11, 12, 45) and, in recent years, by gene cloning and sequencing (5, 10, 13, 16, 28, 41). Although some experimental evidence indicating that these factors are involved in virulence has been obtained (19, 21, 42), their exact role in the pathogenicity of *A. pleuropneumoniae* has not been assessed so far.

The analysis of mutants affected in the expression of specific genes is perhaps the most powerful approach to investigate the role and the mechanism of action of molecules possibly involved in pathogenicity and virulence. To date, only spontaneously occurring or chemically induced *A. pleuropneumoniae* mutants, defective in capsule or hemolysin production, have been studied (1, 21, 22, 35). However, since these mutants are not well characterized genetically (in the sense that the kind and number of genetic determinants affected remain unknown), virulence studies based on them did not provide meaningful information. When genetic systems are not sufficiently developed for a certain bacterial species, as is the case for *A. pleuropneumoniae*, transposon mutagenesis could be a useful tool to generate well-characterized insertion mutants. Although this approach has been widely used in studies on the virulence of a number of gram-negative and gram-positive pathogenic bacteria (14, 47), to our knowledge, transposon mutagenesis has not been achieved in *A. pleuropneumoniae*. In this article, we describe a transposon mutagenesis system that allows the generation, with suitable frequencies, of single, stable, and

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source (reference)
E. coli		
LE392	*supF supE hsdR galK trpR metB lacY tonA*	V. de Lorenzo
CC118(λ*pir*)	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir*	V. de Lorenzo (17)
SM10(λ*pir*)	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc^r::Mu Km^r λ*pir*	M. Simonet
S17-1(λ*pir*)	*thi pro hsd*(r$^-$ m$^+$) *recA*::RP4-2-Tc^r::Mu Km^r::Tn*7* Tp^r Sm^r λ*pir*	V. de Lorenzo
A. pleuropneumoniae[a]		
CM5	Serotype 1	S. Rosendal (34)
ATCC 27088	Serotype 1, reference strain (also known as Shope 4074)	Collection[b]
ATCC 27089	Serotype 2, reference strain (also known as 1536)	Collection
ATCC 27090	Serotype 3, reference strain (also known as 1421)	Collection
ATCC 33377	Serotype 5a, reference strain (also known as K17)	Collection
ATCC 33590	Serotype 6, reference strain (also known as Femϕ)	Collection
WF83	Serotype 7, reference strain	R. Nielsen
405	Serotype 8, reference strain	R. Nielsen
CVJ13261	Serotype 9, reference strain	R. Nielsen
D13039	Serotype 10, reference strain	R. Nielsen
56153	Serotype 11, reference strain	R. Nielsen
G58	Serotype 7, clinical isolate	Our laboratory (15)
G72	Serotype 4, clinical isolate	Our laboratory (15)
Plasmids		
RP4		V. de Lorenzo
pJM703.1::Tn*5*	*oriR6K mobRP4* Ap^r, with Tn*5* (Tn*5*-based delivery plasmid)	M. Simonet
pLOF/Km	Ap^r (Tn*10*-based delivery plasmid, Km^r)	V. de Lorenzo (17)

[a] Nalidixic acid-resistant spontaneous mutants were obtained from all these *A. pleuropneumoniae* strains.
[b] ATCC.

random insertional disruptions in the genome of this bacterium.

Bacterial strains and plasmids. The bacteria and plasmids used in this study are listed in Table 1. *A. pleuropneumoniae* CM5 (serotype 1) (34), a strain that has been used in several investigations on the virulence and immunogenic properties of *A. pleuropneumoniae* (35, 36), was used to develop the transposon mutagenesis system. This strain has no detectable plasmids, as determined by us (not shown) and others (35). For mutagenesis experiments, spontaneously occurring nalidixic acid-resistant mutants of the *A. pleuropneumoniae* strains were obtained.

Culture media and growth conditions. Tryptic soy (TS) broth and TS agar were used for routine cultures. For *A. pleuropneumoniae*, these media were supplemented with 6 mg of yeast extract and 10 μg of NAD (Sigma Chemical Co.) per ml. Antibiotics were added to TS media as required at the following concentrations: ampicillin, 150 μg/ml; kanamycin, 50 μg/ml; tetracycline, 50 μg/ml; and nalidixic acid, 60 μg/ml. For tryptophan auxotrophy experiments, the chemically defined medium of Maudsley and Kadis (27) solidified with 1.5% agar was used. This medium was modified to contain the same concentration of NAD as described above and was supplemented when appropriate with tryptophan (50 μg/ml).

DNA techniques. Plasmid DNA was prepared by using a kit from Stratagene (La Jolla, Calif.). Chromosomal DNA was obtained from *A. pleuropneumoniae* by a simplified lysis procedure with proteinase K and sodium dodecyl sulfate (SDS) (48). For Southern blots (43), chromosomal DNA was digested with *Xho*I as indicated by the manufacturer (New England BioLabs, Inc., Beverly, Mass.), electrophoretically separated on 0.8% agarose gels, and transferred to a nitrocellulose filter (Schleicher & Schuell Inc., Dassel, Germany) as previously described (37). The probe was pLOF/Km radiolabelled by random priming (kit from Amersham Corp., Buckinghamshire, England) with [α-^{32}P]dCTP (Amersham). Hybridization was done under stringent conditions, as follows: prehybridization at 65°C for 4 h with 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS–0.05% skim milk; hybridization overnight at 65°C in the same solution but with only 0.1% SDS; two 30-min washes at room temperature with 2× SSC–0.1% SDS; and two additional 45-min washes at 68°C in 0.2× SSC–0.1% SDS. Filters were then exposed to Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.) for 24 h at −70°C with an amplifying screen.

Conjugation and transposition in *A. pleuropneumoniae*. Transposon mutagenesis in gram-negative bacteria other than *Escherichia coli* is usually achieved with carrier plasmid vectors mobilizable by the conjugal properties of the broad-host-range plasmid RP4 (39). Since there was scarce information about the conjugal properties of *A. pleuropneumoniae*, a first step in our investigation was the determination of the ability of the RP4 plasmid to transfer from *E. coli* LE392 to *A. pleuropneumoniae* CM5. We observed that *A. pleuropneumoniae* CM5 and *E. coli* conjugate, with a frequency of RP4 mobilization in the range of 10^{-4} (Table 2). RP4-mediated tetracycline resistance was not expressed in *A. pleuropneumoniae* CM5, so conjugal transfer events were selected with ampicillin and kanamycin.

The results obtained prompted us to approach transposon mutagenesis in *A. pleuropneumoniae* by the use of plasmids mobilizable through RP4-based conjugal transfer (29). We first used the suicide plasmid pJM703.1::Tn*5*, obtained by M. Simonet and S. Falkow (40) from pJM703.1 (29), with *E. coli* SM10(λ*pir*) as the donor strain. pJM703.1 and its derivatives replicate only in specific bacterial strains, used as donors, producing the π protein determined by the *pir* gene from the plasmid R6K (29). Although this insertional mutagenesis system has proven to be efficient for other bacteria, such as *Yersinia pseudotuberculosis* (40) and *Vibrio cholerae* (29), we have been unable to detect Tn*5* transposition in *A. pleuropneumoniae* CM5.

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TABLE 2. Conjugation and transposition experiments in *A. pleuropneumoniae* CM5[a]

Donor strain (plasmid)	Selection	Resistance frequency
None[b]	Km^r	$<5 \times 10^{-8}$
LE392 (RP4)	Ap^r Km^r	2×10^{-4}
SM10 (pJM703.1::Tn*5*)	Km^r	$<5 \times 10^{-8}$
S17-1 (pLOF/Km), with IPTG	Km^r	1×10^{-5}
S17-1 (pLOF/Km), without IPTG	Km^r	$<5 \times 10^{-8}$

[a] Plasmids were mobilized from *E. coli* donor strains to *A. pleuropneumoniae* by conjugal transfer by means of a filter mating technique, as described previously (7). In brief, 100 μl each of overnight cultures of donor and recipient strains were mixed in 5 ml of sterile 10 mM $MgSO_4$ and then passed through a Millipore type HA 0.45-μm filter. Mating filters containing donor and recipient bacteria were placed onto a TS agar plate (containing 100 μM IPTG when needed for *ptac* induction in the case of the Tn*10* derivative). After incubation at 37°C for 8 h, the bacterial cells were suspended in sterile 0.05 M phosphate buffer (pH 7.4) containing 0.85% NaCl, and appropriate dilutions were plated on the counterselecting medium. Frequencies were determined as the ratio of recipient cells expressing plasmid or transposon antibiotic markers to the total number of recipients.

[b] Unmated recipient. Resistance frequency corresponds to spontaneous Km^r mutants.

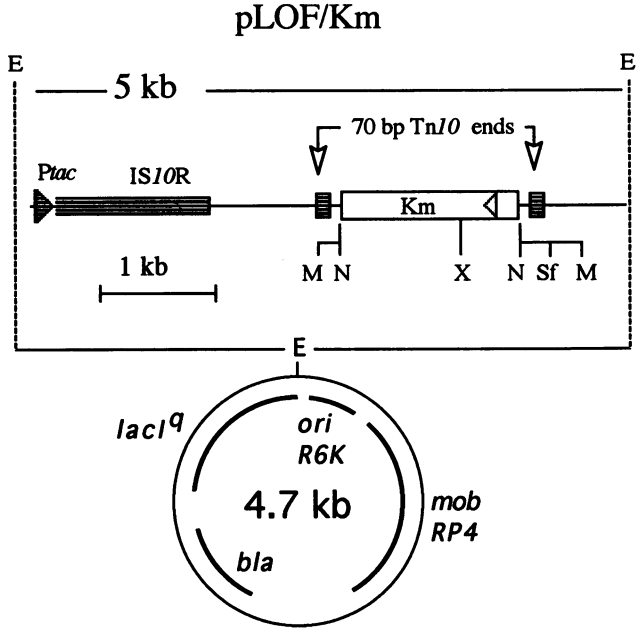

FIG. 1. Design of pLOF/Km, the delivery system for the mini-Tn*10* that is functional in *A. pleuropneumoniae* (adapted from Herrero et al. [17], with permission). Restriction sites are indicated (M, *Mlu*I; N, *Not*I; E, *Eco*RI; Sf, *Sfi*I; X, *Xho*I). The unique *Sfi*I site can be used for the insertion of foreign DNA fragments to be transposed. The *Xho*I site present in the mobile element can be used for cloning the DNA region flanking the mini-Tn*10* insertion from transposon mutants.

Without prejudice that the frequency at which an element transposes is dependent on a great number of factors acting on the system, according to previously reported Tn*5* transposition rates ranging from 10^{-5} to 10^{-6} (6), we can speculate that our negative results are due to the loss of efficiency derived from the low frequency of RP4-mediated conjugal transfer from *E. coli* to *A. pleuropneumoniae* CM5 (10^{-4}) (Table 2). Following this interpretation, the final efficiency rate of mutagenesis (mobilization plus transposition) was so low that Tn*5* insertion mutants were undetectable in practice. In favor of this hypothesis is the fact that, in an identical system involving donor strain *E. coli* SM10 and plasmid pSUP2011, from which pJM703.1 is derived (29), the transfer frequency of Tn*5* into the *Rhizobium meliloti* genome was 5×10^{-5} per recipient, being 10^{-1} per recipient the rate of RP4 mobilization between the same bacteria (39). An additional interpretation that might account for the lack of detection of Tn*5* insertion mutants is the fact that the kanamycin resistance (Km^r) gene of Tn*5* has been reported not to be expressed in *A. pleuropneumoniae* (49).

In view of these problems, we decided to try another conjugative suicide vector, pLOF/Km, constructed by Herrero et al. (17). This is a derivative of pGP704 (pJM703.1 with a polylinker) (29), containing an insert with a mini-Tn*10* (which carries a Km^r cassette derived from Tn*903*) initially designed by Way et al. (46). In the pLOF/Km Tn*10*-based delivery system, the transposase gene of $IS10_R$ is located outside of, and provided in *cis* to, the mobile element. In addition, the transposase gene is under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *ptac* promoter (17), a strategy which could improve transposition efficiency (Fig. 1). When this vector was conjugally transferred from *E. coli* S17-1(λ*pir*) to *A. pleuropneumoniae* CM5 in the presence of IPTG, we found transposition mutants with an efficiency of 10^{-5} (Table 2). In contrast, when IPTG was absent from the mating medium, no mutants were detected, indicating that the transposase expression level is a limiting factor for transposition of the Tn*10* element in the system under study. This result is consistent with the fact that in natural conditions (i.e., without overexpression of the transposase) the IS*10* transposition rate is reported to be about 10^{-4} per element per generation (24), and supports the aforementioned argument explaining our failure in detecting Tn*5* mutants with the pJM703.1::Tn*5* delivery system.

Mutagenesis of *A. pleuropneumoniae* CM5 with the Tn*10* derivative. The transposition properties of the mini-Tn*10*/Km^r in *A. pleuropneumoniae* were investigated further in order to determine the usefulness of the system for insertional mutagenesis in this bacterium. Cointegration of pLOF/Km was determined by selecting for kanamycin resistance (mini-Tn*10*) and ampicillin resistance (determined by the suicide plasmid). We found cointegration rates of about 1%, e.g., 13 of 1,100 colonies were resistant to both antibiotics (1.2% of cointegrates). This frequency is lower than that reported by Herrero et al. (17), who found 5% cointegrates with pLOF/Km in *Pseudomonas putida*, and is much lower than the 35% found by other investigators (3) in *Proteus mirabilis* with a mini-Tn*5* delivery system also derived from pGP704 (7, 29).

A critical parameter in evaluating a transposon mutagenesis system is the ability to produce single, random insertions. To analyze these characteristics, chromosomal DNA from 25 exconjugants from 25 independent mutagenesis experiments was restricted with *Xho*I (which cuts at a unique site in the Tn*10* derivative) (Fig. 1) (17) and analyzed by Southern blotting with pLOF/Km as the probe. Figure 2, in which 19 of these mutants are shown, illustrates that almost all the insertions were single and occurred at different locations. Of the 25 mutants, the insertions in only 2 mapped identically, and in another, a multiple insertion likely took place (Fig. 2). These findings allow to assume that no significant mini-Tn*10* hot spots occur in the *A. pleuropneumoniae* chromosome. This might be explained by the fact that the Tn*10* derivative bears a mutant IS*10* transposase

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with a much lower degree of target site specificity than the wild type (25). Since four of the six consensus base pairs of the target sites of Tn*10* insertion are G-C pairs (25), the relatively low G+C content of the *A. pleuropneumoniae* genome (42%) (23, 32) may also contribute to explaining the absence of significant hot spots.

The insertion randomness of the mini-Tn*10* was also demonstrated by indirect evidence, as provided by the ability to isolate mutants of selected phenotypes. For this purpose, we assessed the frequency of auxotrophic dependence on the amino acid tryptophan in a bank of mini-Tn*10*-induced *A. pleuropneumoniae* mutants. In a prototypic experiment carried out with 1,136 insertional mutants (randomly selected from 10 different mutagenesis experiments), 4 of them were tryptophan auxotrophs. This represents a frequency of about 0.35%, a result in agreement with data on transposon-induced auxotrophic mutations in other bacteria (3, 26). The production of mutants deficient in the expression of other phenotypes was also tested. For instance, we isolated seven nonhemolytic mutants of *A. pleuropneumoniae* (44), indicating that not only metabolic but also virulence-associated genes could be insertionally disrupted by the use of pLOF/Km.

Finally, we carried out experiments to determine the stability of the transposon insertions. By a previously published protocol (3), we subcultured 550 Km^r colonies, selected randomly from five different transposition experiments, three times on TS agar without antibiotics at 37°C for 24 h. After these three passages without selective pressure, the colonies were cultured again on a medium containing kanamycin. None of the 550 clones failed to grow, indicating that the mini-Tn*10* insertions were stably maintained. This result is consistent with the fact that the transposase gene is absent from the mobile element (see above), providing highly stable insertions even without antibiotic selection (17, 25).

Applicability of the mini-Tn*10* transposon mutagenesis system to other *A. pleuropneumoniae* strains. In order to determine whether the transposon mutagenesis system described in this article is of general applicability to the species *A. pleuropneumoniae*, we carried out experiments with 12 additional strains representing different serotypes (Table 1). We were able to obtain nalidixic acid-resistant spontaneous mutants of all these *A. pleuropneumoniae* strains. With the abovementioned *E. coli* donor strain, Km^r exconjugants were found with frequencies ranging from 10^{-5} to 10^{-7} (Table 3). We failed to detect Km^r colonies for only 2 of the 12 strains we tested.

Concluding remarks. We have successfully developed, for the first time, a transposon mutagenesis system that is functional in *A. pleuropneumoniae*. This was achieved with a suicide conjugative vector, pLOF/Km, designed by Herrero et al. (17), carrying a mini-Tn*10* delivery system. In our hands, this vector has proven to be a useful tool for generating single, random, and stable transposon insertions, at a workable frequency, in the *A. pleuropneumoniae* genome. Since the transposable element in transposon-induced mutants is physically linked to the mutated gene, the transposon mutagenesis technique reported here will allow easy identification of the genetic determinants involved in the investigated phenotypes of *A. pleuropneumoniae*. For this purpose, the drug resistance marker of the transposon could also be used for the selection of recombinant clones carrying the target region. Besides these general advantages of transposon mutagenesis, specific benefits derived from the particular features of the vector we used have to be considered.

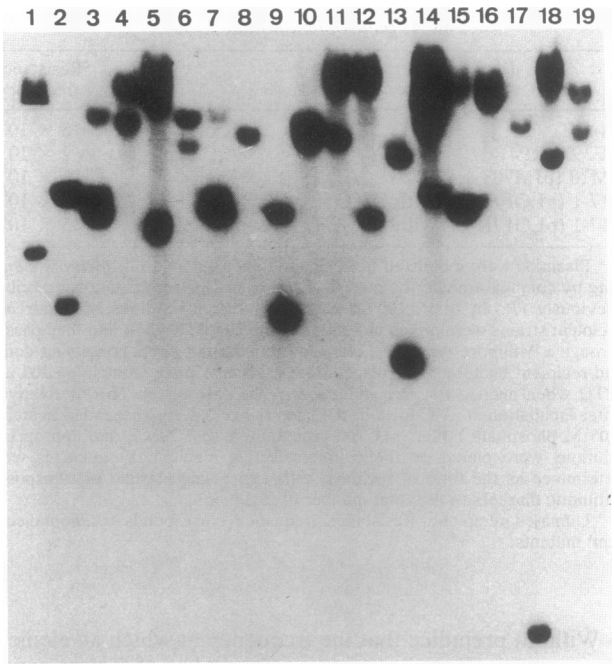

FIG. 2. Random insertion of the Tn*10* element in the *A. pleuropneumoniae* CM5 chromosome, as determined by Southern blot analysis. Chromosomal DNA from 19 kanamycin-resistant, ampicillin-sensitive transconjugants of *A. pleuropneumoniae* (from independent transposition experiments; see text) was digested with *Xho*I, electrophoresed on an agarose gel, transferred to a nylon membrane, and hybridized with a radioactive probe prepared with pLOF/Km to detect DNA fragments containing mini-Tn*10* insertions. Since there was a unique *Xho*I site internal to the mini-Tn*10* element, single-insertion mutants should lead to two reactive bands in the Southern blot hybridization. The figure shows that most of the mutants have single insertions of the mini-Tn*10*, mapping at different sites. Lanes 3 and 7 correspond to mutants with mini-Tn*10* insertions mapping at similar or identical locations. Lane 14 most likely corresponds to a mutant with multiple insertions of the minitransposon.

First, pLOF vectors carry a minitransposon which has important advantages over classical transposable elements (25). Since the transposase gene is located outside the minitransposon, the mobile element does not undergo secondary transposition events, and thus the insertions generated are highly stable. On the other hand, a smaller minitransposon is more manageable, it transposes with an increased efficiency, and, once inserted, it is less subject to rearrangements with adjacent regions (25). Second, the presence of unique *Not*I or *Sfi*I sites internal to the mobile element (17) allows, conjointly with the use of the auxiliary cloning plasmids pUC18Not and pUC18Sfi (specifically constructed for this purpose) (17), the introduction of modified or foreign genes into the genome of the mutated *A. pleuropneumoniae* strain. Third, the modular conception of the mobile element in pLOF vectors (17) enables the replacement of the selection marker. This constitutes an important application which allows the use of nonantibiotic markers (e.g., resistance cassettes to the herbicide bialaphos, to arsenite or mercuric salts, or to organomercurial compounds [17]), opening the possibility of environmental release of the genetically engineered strain, e.g., in the case of a live

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TABLE 3. Transposon mutagenesis of different *A. pleuropneumoniae* strains using pLOF/Km[a]

Recipient strain	Frequency of Km[r] exconjugants
ATCC 27088	1.2×10^{-6}
ATCC 27089	1.3×10^{-5}
ATCC 27090	ND[b]
ATCC 33377	1.1×10^{-6}
ATCC 33590	2.5×10^{-6}
WF83	1.6×10^{-6}
405	1.0×10^{-7}
CVJ13261	1.4×10^{-6}
D13039	3.0×10^{-7}
56153	2.0×10^{-7}
G58	ND
G72	3.3×10^{-6}

[a] Matings were performed and frequencies were determined as indicated in Table 2, footnote *a*.
[b] ND, not detected.

vaccine. Finally, the possibility of replacing the selection marker and the fact that recipient cells are not immune to new transposition runs because of the lack of the transposase permit multiple transposon insertions in the same strain (17).

In conclusion, because of the enormous utility of insertional mutagenesis with transposons in genetic studies of bacteria (4), the availability of such a system for *A. pleuropneumoniae* will contribute greatly to the development of research on molecular aspects of the structure, metabolism, physiology, and virulence of this pathogenic bacterium.

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REFERENCES

1. **Anderson, C., A. A. Potter, and G.-F. Gerlach.** 1991. Isolation and molecular characterization of spontaneously occurring cytolysin-negative mutants of *Actinobacillus pleuropneumoniae* serotype 7. Infect. Immun. **59:**4110–4116.
2. **Bélanger, M., D. Dubreuil, J. Harel, C. Girard, and M. Jacques.** 1990. Role of lipopolysaccharides in adherence of *Actinobacillus pleuropneumoniae* to porcine tracheal rings. Infect. Immun. **58:**3523–3530.
3. **Belas, R., D. Erkshine, and D. Flaherty.** 1991. Transposon mutagenesis in *Proteus mirabilis*. J. Bacteriol. **173:**6289–6293.
4. **Berg, C. M., D. E. Berg, and E. A. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–925. *In* D. E. Berg, and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
5. **Chang, Y.-F., R. Young, and D. K. Struck.** 1991. The *Actinobacillus pleuropneumoniae* hemolysin determinant: unlinked *appCA* and *appBD* loci flanked by pseudogenes. J. Bacteriol. **173:**5151–5158.
6. **de Brujin, F. J., and J. R. Lupski.** 1984. The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids—a review. Gene **27:**131–149.
7. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
8. **Fenwick, B. W., and B. Y. Osburn.** 1986. Immune responses to the lipopolysaccharides and capsular polysaccharides of *Haemophilus pleuropneumoniae* in convalescent and immunized pigs. Infect. Immun. **54:**575–582.
9. **Frey, J., J.-B. Deillon, D. Gygi, and J. Nicolet.** 1991. Identification and partial characterization of the hemolysin (HlyII) of *Actinobacillus pleuropneumoniae* serotype 2. Vet. Microbiol. **28:**303–312.
10. **Frey, J., R. Meier, D. Gygi, and J. Nicolet.** 1991. Nucleotide sequence of the hemolysin I gene from *Actinobacillus pleuropneumoniae*. Infect. Immun. **59:**3026–3032.
11. **Frey, J., and J. Nicolet.** 1988. Purification and partial characterization of a hemolysin produced by *Actinobacillus pleuropneumoniae* type strain 4074. FEMS Microbiol. Lett. **55:**41–46.
12. **Frey, J., and J. Nicolet.** 1991. Immunological properties of *Actinobacillus pleuropneumoniae* hemolysin I. Vet. Microbiol. **28:**61–73.
13. **Frey, J., H. van de Bosch, R. Segers, and J. Nicolet.** 1992. Identification of a second hemolysin (HlyII) in *Actinobacillus pleuropneumoniae* serotype 1 and expression of the gene in *Escherichia coli*. Infect. Immun. **60:**1671–1676.
14. **Gaillard, J.-L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52:**50–55.
15. **Gutiérrez, C. B., R. I. Tascón, J. I. Rodríguez Barbosa, O. R. González, J. A. Vázquez Boland, and E. F. Rodríguez-Ferri.** 1993. Characterization of V factor-dependent organisms of the family *Pasteurellaceae* isolated from porcine pneumonic lungs in Spain. Comp. Immunol. Microbiol. Infect. Dis. **16:**123–130.
16. **Gygi, D., J. Nicolet, J. Frey, M. Cross, V. Koronakis, and C. Hughes.** 1990. Isolation of the *Actinobacillus pleuropneumoniae* haemolysin gene and the activation and secretion of the prohaemolysin by the HlyC, HlyB and HlyD proteins of Escherichia coli. Mol. Microbiol. **4:**123–128.
17. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertions of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
18. **Higgins, R., S. Larivière, K. R. Mittal, G. P. Marineau, P. Rousseau, and J. Cameron.** 1985. Evaluation of a killed vaccine against porcine pleuropneumonia due to *Haemophilus pleuropneumoniae*. Can. Vet. J. **26:**86–89.
19. **Inzana, T. J.** 1991. Virulence properties of *Actinobacillus pleuropneumoniae*. Microb. Pathogen. **11:**305–316.
20. **Inzana, T. J., and B. Mathison.** 1987. Serotype specificity and immunogenicity of the capsular polymer of *Haemophilus pleuropneumoniae* serotype 5. Infect. Immun. **55:**1580–1587.
21. **Inzana, T. J., J. Todd, J. Ma, and H. Veit.** 1991. Characterization of a nonhemolytic mutant of *Actinobacillus pleuropneumoniae* serotype 5: role of the 110-kilodalton hemolysin in virulence and immunoprotection. Microb. Pathogen. **10:**281–296.
22. **Inzana, T. J., J. Todd, and H. P. Veit.** 1993. Safety, stability, and efficacy of noncapsulated mutants of *Actinobacillus pleuropneumoniae* for use in live vaccines. Infect. Immun. **61:**1682–1686.
23. **Kilian, M., and E. L. Biberstein.** 1984. Genus II. Haemophilus, p. 558–569. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
24. **Kleckner, N.** 1989. Transposon Tn*10*, p. 227–268. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
25. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
26. **Leyh, R. D., R. W. Griffith, and L. H. Arp.** 1988. Transposon mutagenesis in *Bordetella avium*. Am. J. Vet. Res. **49:**687–692.
27. **Maudsley, J. R., and S. Kadis.** 1986. Growth and hemolysin production by *Haemophilus pleuropneumoniae* cultivated in a chemically defined medium. Can. J. Microbiol. **32:**801–805.

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28. **McWhinney, D. R., Y.-F. Chang, R. Young, and D. K. Struck.** 1992. Separable domains define target cell specificities of an RTX hemolysin from *Actinobacillus pleuropneumoniae*. J. Bacteriol. **174**:291–297.
29. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in constructions of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170**:2575–2583.
30. **Nicolet, J.** 1986. *Haemophilus* infections, p. 426–436. *In* A. D. Leman, B. Straw, R. D. Glock, W. L. Mengeling, R. H. C. Penny, and E. Scholl (ed.), Diseases of swine. Iowa State University Press, Ames, Iowa.
31. **Nielsen, R.** 1986. Serological characterization of *Actinobacillus pleuropneumoniae* strains and proposal of a new serotype: serotype 12. Acta Vet. Scand. **27**:452–455.
32. **Pohl, S., H. U. Berstchinger, W. Frederiksen, and W. Mannheim.** 1983. Transfer of *Haemophilus pleuropneumoniae* and the *Pasteurella haemolytica*-like organism causing porcine necrotic pleuropneumonia to the genus *Actinobacillus* (*Actinobacillus pleuropneumoniae* com. nov.) on the basis of phenotypic and deoxyribonucleic acid relatedness. Int. J. Syst. Bacteriol. **33**:510–514.
33. **Rapp, V. J., and R. F. Ross.** 1986. Antibody response of swine to outer membrane components of *Haemophilus pleuropneumoniae* during infection. Infect. Immun. **54**:751–760.
34. **Rosendal, S., D. S. Carpenter, W. R. Mitchell, and M. R. Wilson.** 1981. Vaccination against pleuropneumonia of pigs caused by *Haemophilus pleuropneumoniae*. Can. Vet. J. **22**:34–35.
35. **Rosendal, S., and J. I. MacInnes.** 1990. Characterization of an attenuated strain of *Actinobacillus pleuropneumoniae*, serotype 1. Am. J. Vet. Res. **51**:711–717.
36. **Rosendal, S., O. P. Miniats, and P. Sinclair.** 1986. Protective efficacy of capsule extracts of *Haemophilus pleuropneumoniae* in pigs and mice. Vet. Microbiol. **12**:229–240.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Sebunya, T. N. K., and J. R. Saunders.** 1983. *Haemophilus pleuropneumoniae* infections in swine: a review. J. Am. Vet. Med. Assoc. **182**:1331–1337.
39. **Simon, R., U. Priefer, and A. Pühler.** 1982. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1**:784–791.
40. **Simonet, M., and S. Falkow.** 1991. Unpublished results.
41. **Smits, M. A., J. Briaire, R. Jansen, H. E. Smith, E. M. Kamp, and A. L. J. Gielkens.** 1991. Cytolysins of *Actinobacillus pleuropneumoniae* serotype 9. Infect. Immun. **59**:4497–4504.
42. **Smits, M., E. Kamp, J. Briaire, R. Jansen, L. van Leengoed, and J. van Dijk.** 1992. Induction of pneumonic lesions by recombinant cytolysins of *Actinobacillus pleuropneumoniae*, abstr. 187. Proc. 12th IPVS Congress, The Hague, The Netherlands.
43. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
44. **Tascón, R. I., E. F. Rodríguez-Ferri, J. A. Vázquez-Boland, et al.** Unpublished data.
45. **Thwaits, R. N., and S. Kadis.** 1991. Immunogenicity of *Actinobacillus pleuropneumoniae* outer membrane proteins and enhancement of phagocytosis by antibodies to the proteins. Infect. Immun. **59**:544–549.
46. **Way, J. C., M. A. Davis, D. Morisato, D. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32**:369–379.
47. **Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow.** 1983. Tn*5*-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. **42**:33–41.
48. **Wilson, K.** 1990. Preparation of genomic DNA from bacteria: miniprep of bacterial genomic DNA, p. 1–2, unit 2.4.1. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
49. **Wilson, P. J.** 1990. *Haemophilus*, *Actinobacillus*, *Pasteurella*: mechanisms of resistance and antibiotic therapy. Can. J. Vet. Res. **54**:S73–S77.

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